J. Microbiol. Biotechnol. (2008), **18**(1), 107–109

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Peroxidase-mediated Formation of the Fungal Polyphenol 3,14'-Bihispidinyl

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Received: May 27, 2007 / Accepted: July 20, 2007

Medicinal fungi, *Phellinus linteus* and *Inonotus xeranticus*, produce a cluster of yellow pigment in their fermentation broth that acts as an important element of biological activity. The pigment is composed of diverse polyphenols with a styrylpyrone moiety, mainly hispidin and its dimers, 3,14'-bihispidinyl, hypholomine B, and 1,1-distyrylpyrylethan. Although dimeric hispidins were proposed to be biosynthesized from two molecules of monomer *via* oxidative coupling by ligninolytic enzymes, laccase and peroxidase, the details of this process remain unknown. In this preliminary study, we attempted to achieve enzymatic synthesis of the hispidin dimer from hispidin by using commercially available horseradish peroxidase (HRP). Consequently, a hispidin dimer, 3,14'-bihispidinyl, was synthesized, whereas the other dimers, hypholomine B and 1,1-distyrylpyrylethan, were not produced. This result suggested that the oxidative coupling at the C-3 and C-14' positions of hispidins was dominant in the process of dimerization by HRP, and indicated that additional catalysts or substrates would be needed to synthesize other hispidin dimers present in the fungal metabolite.

Keywords: Enzymatic synthesis, hispidin, hispidin dimers, 3,14'-bihispidinyl, peroxidase-mediated dimerization

Mushrooms are ubiquitous in nature and produce various classes of secondary metabolites with interesting biological activities [6, 10]. The medicinal mushrooms, such as *Phellinus linteus*, *P. baumii*, *Inonotus obliquus*, and *I. xeranticus*, have been used as traditional medicines for the treatment of various diseases, including stomach ailments, diabetes, gastroenteric disorders, lymphatic diseases, and cancers in Korea, China, Japan, and other Asian countries [14, 16]. Interestingly, the mycelial cultures of these medicinal fungi produce a yellow antioxidant pigment that is composed of styrylpyrone class analogs. Hispidin, a major constituent, was previously isolated from the fruiting bodies of *Inonotus hispidus* as a selective PKC-B inhibitor [5] and an antiviral agent [2]. In addition, hispidin and its dimers, hypholomine B and 3,14'-bihispidinyl, are important elements in the genera *Phellinus* and *Inonotus*, and thus, their co-occurrence has been used in the chemotaxonomic study of *Phellinus* and *Inonotus* spp. [3].

Recently, we found that the fruiting body of *I. xeranticus* contained diverse hispidin derivatives with potent antioxidant activity [8, 9, 11, 12], and also determined that its mycelial culture produced hispidin and its dimers, 3,14'-bihispidinyl, hypholomine B, and 1,1-distyrylpyrylethan. In terms of free-radical scavenging activity, hispidin dimers exhibited higher activity than the hispidin monomer. The dimeric hispidin has been biogenetically regarded as a condensation product of dehydrohispidin [4, 7]. It is known that many phenolic compounds were transformed into polymeric structures by the ligninolytic enzymes, laccase and peroxidase, which were secreted by various white rot fungi [15]. Although dimeric hispidins have been proposed to be biosynthesized by the oxidative coupling of two hispidin molecules, the details of this process remain unknown. In this preliminary study, we attempted to achieve enzymatic synthesis of hispidin dimers from the synthesized hispidin by using commercially available horseradish peroxidase (HRP).

Synthesis of Hispidin

To verify the dimerization of hispidin by peroxidase, hispidin was synthesized using a previously reported method [1] with some modification. A suspension of $Mg(OMe)_2$ (10 mmol) in anhydrous MeOH (10 ml) was added into a solution of piperonal (3 mmol) and 4-methoxy-6-methyl-2-pyrone (2.4 mmol) in MeOH (8 ml) under N_2 gas. The mixture was refluxed for 7 h, and the solvent was removed by evaporation. The residue was subjected to a column of silica gel eluted with $CHCl_3$-MeOH=50:1–10:1 to afford 220 mg (35% of yield) of 4-methoxy-6-(3',4'-methylenedioxystyryl)-2-pyrone. A solution of this compound (0.8 mmol) in CH_2Cl_2 (20 ml) was added to a 1 M solution of BCl_3 in hexane (2.5 mmol) under N_2 gas. The reaction mixture was refluxed for 22 h. The

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solvent was removed, and the residue was subjected to a column of silica gel eluted with $CHCl_3$-MeOH=30:1–2:1 to afford 180 mg (90% of yield) of 4-methoxy-6-(3',4'-dihydroxystyryl)-2-pyrone. A solution of EtSH (1.4 mmol) in 4 ml of DMF was added to a suspension of NaH (15 mg, 60% oil suspension) in 2 ml of DMF under N_2 gas. The mixture was stirred for 10 min, and a solution of 4-methoxy-6-(3',4'-dihydroxystyryl)-2-pyrone (0.4 mmol) in 2 ml of DMF was then added. The reaction mixture was refluxed for 1 h. The crude product was chromatographed on a Sephadex LH-20 column eluting with 70% aqueous MeOH to yield 49 mg (50% of yield) of hispidin in the form of a yellow powder. The structure of synthesized hispidin was confirmed by comparing its ^{1}H-NMR data with that described previously [1].

Peroxidase-catalyzed Dimerization of Hispidin

The hispidin dimers present in the fermentation broth of medicinal fungi were known to be 3,14'-bihispidinyl, hypholomine B, and 1,1-distyrylpyrylethan (Fig. 1). In fact, we cultured a strain of *Phellinus linteus* by using potato dextrose broth medium and analyzed the ethyl acetate extract of the cultured broth by analytical reversed-phase high-performance liquid chromatography (HPLC). For HPLC, a linear gradient using water acidified with 0.04% TFA (v/v) and methanol was used at a flow rate of 1 ml/min after 5 min at 30% methanol, and reached 90% methanol in 23 min. Consequently, the fungus *P. linteus* produced hispidin and its dimers, 3,14'-bihispidinyl, hypholomine B, and 1,1-distyrylpyrylethan, in its fermentation broth (Fig. 2a). Each peak was identified by various NMR spectra and mass measurements (data not shown). In fermentation broth, hispidin, hypholomine B, 1,1-distyrylpyrylethan, and 3,14'-bihispidinyl were produced in order of yield. It has been proposed that these dimeric compounds would be biosynthesized by the oxidative coupling of two molecules of hispidin, and that its reaction would be catalyzed by mushroom peroxidase. To ascertain the dimerization of hispidin by peroxidase, we used a commercially available horseradish peroxidase, which was known to initiate dehydrogenative polymerization of phenolic compounds. Then, 30% H_2O_2 (1 μl) was added dropwise at an interval of 1 min (total 10 μl) to a vigorously stirred solution of synthesized hispidin (40 μmol) and 30 μl of horseradish peroxidase (HRP) (20 U, Sigma-Aldrich Co.) in a mixture of methanol and 0.1 M Tris-HCl buffer (pH 7.0, 1:1 v/v, 10 ml in total). After the mixture was stirred for 4 h at 30°C, the solvent was evaporated under reduced pressure, and the residue was analyzed by analytical reversed-phase HPLC eluted with the same gradient solvent system as that for fermentation broth [13]. The synthesized hispidin gave a peak (Fig. 2b), and a control with no enzyme in the reaction mixture also provided the same HPLC profile as that of Fig. 2b. The reaction with enzyme, however, provided a new peak with a retention time similar to that of compound **2** (Fig. 2c). To confirm the structure, the reactant was subjected to reversed-phase thin-layer chromatography

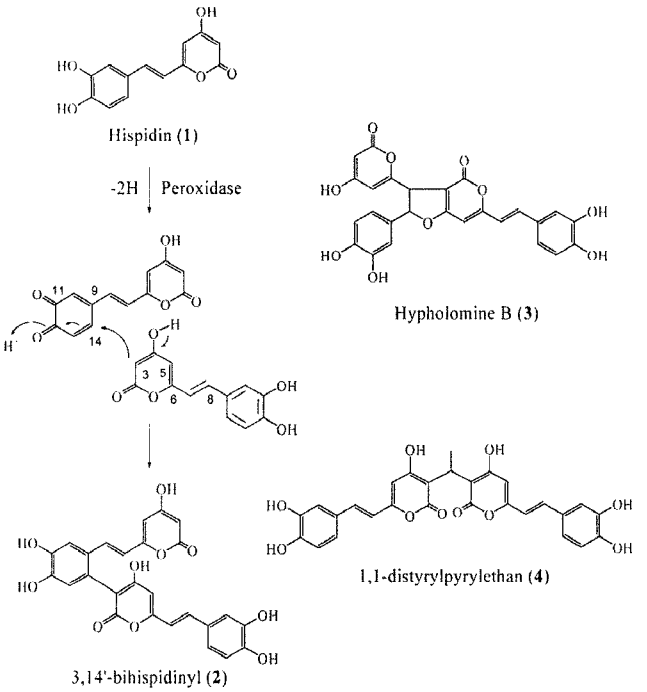

Fig. 1. Structures of hispidin and its dimers, 3,14'-bihispidinyl, hypholomine B, and 1,1-distyrylpyrylethan, and the proposed dimerization mechanism of hispidin into 3,14'-bihispidinyl by horseradish peroxidase.

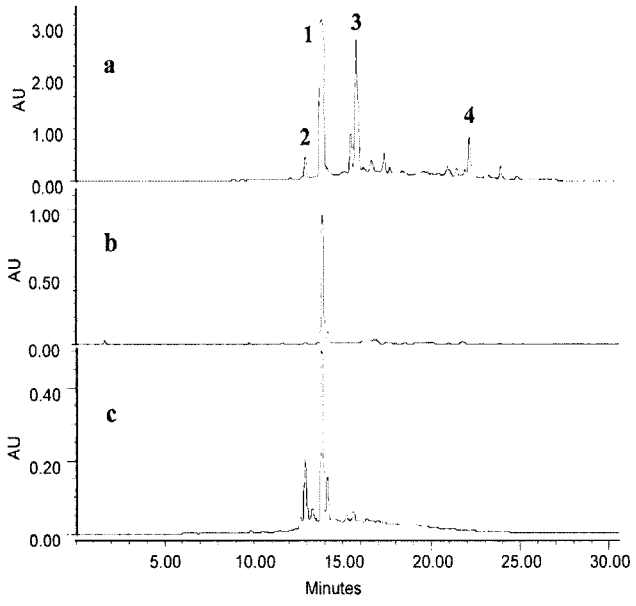

Fig. 2. HPLC profile of (**a**) the antioxidant fraction of the fermentation broth of *Phellinus linteus* 52404 (**1**, hispidin; **2**, 3,14'-bihispidinyl; **3**, hypholomine B; and **4**, 1,1-distyrylpyrylethan), (**b**) synthesized hispidin, and (**c**) the reaction products from incubation of hispidin (40 μmol) and horseradish peroxidase (30 μl in 20 U)/H_2O_2 (10 μl).

(TLC) developed with 50% aqueous methanol, and two compounds, identical to two major peaks with retention times of 12.9 and 13.8 min, were purified. Based on the electrospray ionization (ESI)-mass and ^{1}H-NMR spectra, the two compounds were identified as 3,14'-bihispidinyl and hispidin, respectively [7]. This result revealed the dominance of coupling modes that produce a covalent bond at the C-3 and C-14' of the two hispidins (Fig. 1). On the other hand, no production of hypholomine B (although a small amount of a compound with similar retention time to compound **3** was produced, its UV spectrum was different from that of hypholomine B) or 1,1-distyrylpyrylethan was evident, and it was thought that an additional catalyst or substrate was required to produce these compounds in the fungal metabolite.

This is the first study on HRP-mediated dimerization of hispidin. This result suggests that the hispidin redox potentials and different types of peroxidase are sufficiently accessible to generate the biosynthetic diversity of hispidin and probably to produce many hispidin derivatives that have not yet been identified.

Acknowledgments

This work was supported by a grant (20050401-034-645-196) from the BioGreen 21 Program of the Rural Development Administration and, in part, by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

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